JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2005, p. 4205–4207 Vol. 43, No. 8
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.8.4205–4207.2005
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Use of the MGB Eclipse System and SmartCycler PCR for Differentiation of *Mycobacterium chelonae* and *M. abscessus*

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Received 18 March 2005/Returned for modification 10 May 2005/Accepted 17 May 2005

Although accurate in the identification of *Mycobacterium* species, partial 16S rRNA gene sequencing does not distinguish *Mycobacterium chelonae* from *M. abscessus*. Thus, we designed a SmartCycler PCR assay targeting the 16S-to-23S internal transcribed spacer (ITS) region with use of MGB Eclipse probes to distinguish each species. Comparison with PCR-restriction enzyme analysis of a 441-bp fragment of the *hsp65* gene resulted in 100% correlation with 25 isolates of *M. chelonae* and 25 isolates of *M. abscessus*. ITS PCR performed on 90 consecutive isolates identified by partial 16S rRNA gene sequencing (26 isolates of the *M. chelonae-M. abscessus* complex and 64 remaining isolates, including *Mycobacterium* species, *Nocardia* species, and other aerobic actinomycetes) showed 100% specificity and sensitivity. The ITS PCR assay is accurate and specific, easy to perform, and a good supplemental test when using partial 16S rRNA gene sequencing to identify *M. chelonae* and *M. abscessus*.

The use of partial (5′ end, approximately 500 bp) 16S rRNA gene sequencing is an accurate method for the identification of *Mycobacterium* species from cultured isolates (1, 3, 5, 8, 10, 14). This method, however, does not differentiate *Mycobacterium chelonae* from *M. abscessus*. Conventional tests that have been used to separate these two species include growth on 5% sodium chloride and use of citrate medium as a carbon source (16). Limitations of the conventional tests include difficulty interpreting results (2) and prolonged turnaround time, requiring up to 3 weeks of incubation. Restriction enzyme digestion and analysis of an amplified fragment (441 bp) of the *hsp65* gene can distinguish *M. chelonae* from *M. abscessus* but requires gel analysis and interpretation of fragment sizes and is sometimes difficult because of the similarity of restriction patterns (11). The objective of this study was to develop a simple and rapid method, using real-time PCR with the SmartCycler (Cepheid, Sunnyvale, CA), that would differentiate between *M. abscessus* and *M. chelonae*.

To initiate the design of primers and probes, sequences of the 16S-to-23S internal transcribed spacer (ITS) region of several *Mycobacterium* species were either determined by ARUP laboratories or retrieved from GenBank (http://www.ncbi.nlm.nih.gov). The sequences were subsequently aligned by using Lasergene v6.0 (DNASTAR, Inc., Madison, WI), and conserved and variable regions were determined. Oligonucleotide custom sequences were selected for use with the MGB Eclipse Probe System using the MGB Eclipse Design Software (Nanogen, Inc., Bothell, WA). The primer mix consists of forward primer (5′-CATTTCCCAGTCGAATGAACT) and reverse primer (5′-CAGG[A*]TTTA[A*]AAA[A*]CAT[A*]TTCAC CAAG, where [A*] represents a proprietary modified base, Super A). *M. chelonae* is detected by a tetracycline-labeled probe (5′-AG[N*]G[A*]GTTTC[T*]GT[A*]G[T*]GG where [N*] represents a universal proprietary modified base, Super N, and [A*] and [T*] represent proprietary modified bases Super A and Super T, respectively). *M. abscessus* is detected by a 6-carboxyfluorescein-labeled probe (5′-AGTAGGCATCTG TAGTGG). Table 1 shows the region of probe hybridization among different sequences for three strains of the *M. chelonae* species and two strains of the *M. abscessus* species. The current assay was designed to detect all of these variants.

DNA was extracted from isolated bacterial organisms by using the PrepMan Ultra reagent (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The extracted material was held at 4°C until either analysis by PCR or partial 16S rRNA gene sequence analysis (1 to 8 weeks).

The PCR master mix consisted of 1 μM of each primer and 0.2 μM of each probe added to the appropriate number of OmniMix beads (Cepheid, San Diego, CA) determined by the manufacturer. Per each sample, 4 μl of DNA was added, as was enough water to make the final volume of each PCR mixture 25 μl. After an initial denaturation at 95°C for 2 min, three-step PCR was performed with 42 cycles of 20 s each at 95°C, 55°C, and 76°C followed by a final extension at 76°C for 2 min. The PCR protocol was optimized by testing reference samples (*M. abscessus* ATCC 19977^T and *M. chelonae* ATCC 35752^T) with various concentrations of magnesium (4 μM to 6 μM) and primers (0.5 μM to 1.3 μM) and annealing temperatures ranging from 50°C to 60°C.

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TABLE 1. Probe sequences showing positions of ambiguity among various strains of *M. chelonae* and *M. abscessus*

Probe or species	Source/accession no.	Probe target sequence[a]
M. chelonae PCR probe	Current assay	AGNGAGTTTCTGTAGTGG
M. chelonae	ATCC 35752[T]	AGCGAGTTTCTGTAGTGG
	GenBank AJ291582, AJ291583	AGTGAGTTTCTGTAGTGG
	GenBank AJ291584	AGCGAGTATCTGTAGTGG
M. abscessus PCR probe	Current assay	AGTAGGCATCTGTAGTGG
M. abscessus	ATCC 19977[T]	AGTAGGCATCTGTAGTGG
M. abscessus type II	ATCC 700868, 700869	AGTAGGCATCTGTAGTGG

[a] Underlined bases are positions of ambiguity.

The ability of the assay to differentiate between *M. chelonae* and *M. abscessus* was evaluated in a blinded fashion by testing 50 strains of the *M. chelonae-M. abscessus* complex identified by PCR-restriction enzyme analysis of a 441-bp (Telenti) fragment of the *hsp65* gene as previously described (12, 13). The comparison resulted in 100% agreement between the two methods. One half of the samples were positive for *M. abscessus* (two of which were determined by *hsp65* PCR-restriction enzyme analysis to be type II) (4), and half were positive for *M. chelonae*. The two isolates of *M. abscessus* type II were detected but not separated from *M. abscessus* type I by the ITS PCR assay.

ITS PCR specificity was determined by analysis of *Mycobacterium* species other than *M. chelonae* and *M. abscessus* as well as various *Nocardia* species, other aerobic actinomycetes, and the contaminants *Staphylococcus epidermidis* and *Microbacterium* species. Ninety consecutive samples of mycobacteria and suspected aerobic actinomycetes identified by partial 16S rRNA gene sequencing (Table 2) were tested by the assay. Of the 90 samples tested, 64 were identified by partial 16S rRNA gene sequencing as *Mycobacterium* species other than *M. chelonae* or *M. abscessus* or nonmycobacterium bacterial species, all of which tested negative by ITS PCR (100% specificity).

By partial 16S rRNA gene sequencing, the *M. chelonae-M. abscessus* complex is identified, but differentiation between the two species cannot occur, as the first 500 bp of the sequence are identical. Of 26 consecutive clinical isolates identified by sequencing as part of the *M. chelonae-M. abscessus* complex, 22 were *M. abscessus* isolates and 4 were *M. chelonae* isolates according to the ITS PCR method described here. Our finding that *M. abscessus* occurs more often than *M. chelonae* is consistent with previous studies reported in the literature (6, 7, 15).

The ITS PCR assay cannot distinguish *M. abscessus* type I from *M. abscessus* type II, as the amplicon sequences for each are identical (Table 1). This limitation has no clinical relevance currently for the diagnosis of infection by *M. abscessus* except for the possibility of epidemiological analysis in clinical infection. The most important benefit of the ITS PCR assay is the ability to differentiate between *M. chelonae* and *M. abscessus*, since infections with these organisms require different treatment regimens (16, 17).

The introduction of real-time PCR assays to the clinical microbiology laboratory has led to significant improvements in the diagnosis of infectious disease. There has been steadily increasing interest in this technique, and several hundred reports describing applications in clinical microbiology have been published. The real-time PCR system allows accurate detection of the target sequence in less than 1 h with continuous monitoring of the PCR. The closed-tube format used with the SmartCycler system, which removes the need for postamplification manipulation of the PCR products, reduces the likelihood of amplicon carryover to subsequent reactions and, hence, the risk of false positives.

Proprietary superbases were incorporated into the design of the probe sequence for *M. chelonae* to enable detection of the

TABLE 2. Validation of the ITS PCR assay using clinical isolates: comparison with partial (5′) 16S rRNA gene sequencing

Partial 16S rRNA identification	No. of isolates tested	ITS PCR result
M. chelonae-M. abscessus	22	Positive, *M. abscessus*
M. chelonae-M. abscessus	4	Positive, *M. chelonae*
Mycobacterium branderi	1	Negative
Mycobacterium fortuitum complex sqv[a]		
sqv I	1	Negative
sqv II	1	Negative
sqv III	4	Negative
sqv IV	1	Negative
Mycobacterium goodii	1	Negative
Mycobacterium gordonae sqv[a]		
I	9	Negative
II	1	Negative
III	1	Negative
V	2	Negative
Mycobacterium immunogenum	1	Negative
Mycobacterium intracellulare	3	Negative
Mycobacterium kansasii[b]	1	Negative
M. kansasii (sqv III or VI-2)[a]	1	Negative
Mycobacterium kubicae	2	Negative
Mycobacterium lentiflavum	2	Negative
Mycobacterium marinum[b]	3	Negative
Mycobacterium mucogenicum	3	Negative
Mycobacterium neoaurum	1	Negative
"Mycobacterium paraffinicum"	1	Negative
Mycobacterium parascrofulaceum	1	Negative
Mycobacterium shimoidei	1	Negative
Mycobacterium simiae	3	Negative
Mycobacterium szulgai	1	Negative
Mycobacterium terrae	1	Negative
Mycobacterium terrae complex (MCRO6[c])	1	Negative
Mycobacterium triplex	1	Negative
Mycobacterium xenopi	2	Negative
"Mycobacterium lacticola"	2	Negative
Microbacterium species	1	Negative
Nocardia asteroides (drug group IV)	1	Negative
Nocardia transvalensis	1	Negative
Nocardia cyriacigeorgica	2	Negative
Nocardia farcinica	1	Negative
Nocardia veterana	1	Negative
Arcobacter butzleri	1	Negative
Gordonia bronchialis	1	Negative
Rhodococcus sp.	1	Negative
Staphylococcus epidermidis	1	Negative

[a] Sequevars (sqv) numbered according to RIDOM (http://www.ridom-rdna.de) (9).
[b] Sequence identification confirmed by colony morphology and pigmentation studies.
[c] Matching sequence retrieved from GenBank (http://www.ncbi.nlm.nih.gov).

three different strains with compatible melting temperatures for the PCR parameters. No superbases were used for the probe sequence for *M. abscessus*, since there were no sequence variations among the strains. In this assay, the fluorescence level of the probe with incorporated superbases is lower than that of the probe without incorporated superbases; however, assay optimization allowed appropriate detection of both probes. While it may be possible to develop a multiplex assay using other probe and primer chemistries, it would likely be a challenge without the use of superbase technology utilized by the MGB Eclipse Probe System for the detection and distinction of *M. chelonae* and *M. abscessus*.

The ITS SmartCycler PCR assay described is designed for use in the clinical microbiology laboratory as a component of the culture algorithm for the identification of *Mycobacterium* species. The MGB Eclipse Probe System proved to be an excellent design of the assay with good performance using the SmartCycler instrument. The ITS SmartCycler PCR assay is a good addition to our current *Mycobacterium* species identification algorithm using partial 16S rRNA gene sequencing as the primary identification tool.

This study was supported by the ARUP Institute for Clinical and Experimental Pathology.

We thank Haleina Neal of ARUP Laboratories for her technical assistance.

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